Prognostic Value of Bone Sialoprotein Expression in Clinically Localized Human Prostate Cancer

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Background: Bone sialoprotein (BSP), a bone matrix protein, was recently found to be expressed ectopically in breast cancer and to have a statistically significant association with poor prognosis and the development of bone metastases in that disease. These data prompted us to investigate whether BSP might also be expressed in human prostate cancer, which often metastasizes to bone, and be predictive for progression risk. *Methods:* Tissue sections from 180 patients who had undergone a radical prostatectomy for localized prostate cancer were analyzed immunohistochemically for BSP expression. Biochemical progression was defined as an increasing serum prostate-specific antigen level of 0.5 ng/mL or more. Statistical analysis was used to assess associations between pathologic findings and level of BSP expression, and a Cox proportional hazards model was used to determine which clinical and histologic parameters, including stage, Gleason score, and BSP expression (immunostaining intensity and extent), were independently associated with biochemical progression. All *P* values were two-sided. *Results:* Most of the prostate cancer lesions examined (78.9%) expressed detectable levels of BSP, compared with no or low expression in the adjacent normal glandular tissue. A statistically significant association was found between BSP expression and biochemical progression in both univariate and multivariate analyses. After a follow-up interval of 3 years, the biochemical relapse rate was 36.7% (95% confidence interval [CI] = 23.4%–47.7%) in patients whose tumors expressed high levels of BSP compared with 12.1% (95% CI = 2.3%–20.8%) in patients whose tumors expressed no or a low detectable level of the protein (logrank test, *P* = .0014). BSP expression status could identify those patients at higher risk of biochemical progression (logrank test, *P*<.05) among patients with moderately differentiated tumors or with pathologically confined tumors. *Conclusions:* To our knowledge, this study is the first to demonstrate BSP expression in human prostate cancer and to highlight the protein's statistically significant prognostic value in patients with clinically confined prostate adenocarcinomas. [J Natl Cancer Inst 1998;90:1000–8]

Bone metastases are a major clinical problem that produces significant morbidity. In metastatic prostate cancer, the skeleton is frequently invaded by prostate tumor cells, which are second only to breast cancer in their osteotropism. It is now generally acknowledged that, besides the anatomical or mechanical theories that implicate the rich vascular anastomoses linking the venous vertebral system and the peri-prostatic venous plexus in this tropism *(1)*, several specific biochemical factors involving both the metastatic cell and the host tissue are implicated in the regulation of bone metastasis formation *(2–4)*. Among those factors, the preferential adhesion of prostate carcinoma cells to bone marrow has been proposed as playing a significant role *(5)*.

Bone sialoprotein (BSP) is one of the prominent glycoproteins found in the

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See "Notes" following "References."

mineral compartment of bone, constituting 10%–15% of the noncollagenous bone matrix proteins *(6)*. BSP may play important roles in the regulation of mineralization during bone resorption and formation *(7,8)* and may mediate some interactions between bone cells and the mineralized matrix *(9,10)*. Until recently, BSP expression was thought to be restricted to mineralizing connective tissue, with the exception of decidual cells and trophoblasts of the developing placenta *(11–13)*. Our finding that BSP is ectopically expressed at both the protein and messenger RNA (mRNA) levels in a variety of osteotropic human cancers, such as breast and lung cancers, as well as multiple myeloma, has shed light on potential, important biologic functions for this protein *(6,14,15)*. BSP expression in breast cancer was associated with poor prognosis and, interestingly, with an increased risk that the patient would develop bone metastasis *(16,17)*. Prostate cancer, like breast cancer, belongs to the restricted group of highly osteotropic cancers.

Altogether, these observations prompted us to examine the expression of BSP in prostate cancer. In this study, we have evaluated the expression of BSP in a large series of human prostate adenocarcinomas by immunoperoxidase and *in situ* hybridization techniques. In addition, we have examined the potential prognostic value of the level of BSP expression.

Materials and Methods

Patients and Tissue Specimens

Archival paraffin-embedded tissue samples from human prostate cancers were obtained from the Department of Pathology at the Centre Hospitalier Universitaire de Liège, Belgium. These samples were surgically obtained from 180 patients who had undergone a radical retropubic prostatectomy for localized prostate cancer during the period from 1987 through 1995. No patient who had received prior hormonal therapy, chemotherapy, or radiation therapy was included in the investigation. The ages of the patients and the pathologic stage of their disease are shown in Table 1. All patients had a clinically confined tumor, classified as stage A (n = 44) or B (n = 136) according to the TNM system *(18)*. Absence of regional or distant extension of the tumor was assessed before surgery by chest x ray, pelvic computed tomography scan, and bone scanning. After histopathologic examination, 99 patients were classified as having tumors of pathologic stage pT2. In 56 patients, extracapsular extension of the

Table 1. Characteristics of 180 patients with clinically localized prostate cancer treated with radical retropubic prostatectomy during the period from 1987 through 1995

| | Clinical stage | | | | | | |
| | T1 (Gleason score) | | | T2 (Gleason score) | | | |
	2–4*	5–7†	8–10‡	2–4*	5–7†	8–10‡	Total
No. of patients	13	29	2	21	93	22	180
Age, y							
Mean	65.7	64.9	66.8	65.4	64.9	67.9	65.4
Standard deviation	6.6	6.5	4.4	5.2	5.5	3.3	5.5
Pathologic stage, %							
Organ confined (pT2)	100.0	69.0	50.0	90.4	45.2	18.2	55.0
Extracapsular (pT3A–B)	0	24.1	50.0	4.8	40.8	40.9	31.1
Seminal vesicle invasion (pT3C)	0	6.9	0	4.8	14.0	40.9	13.9

*Well differentiated.
†Moderately differentiated.
‡Poorly differentiated.

tumor (stage pT3A–B) was observed. The remaining 25 patients were categorized as having prostate tumors of stage pT3C because their tumors showed evidence of invading the seminal vesicle. All specimens were evaluated according to the Gleason scoring system *(19)* (Table 1). Results from postoperative monitoring of total serum prostate-specific antigen (PSA) levels were available for 164 of these patients who had been followed for a mean time of 24 months (95% confidence interval [CI] = 20.8–27.0). For the 16 patients who were lost to follow-up, there were no significant differences in clinical or pathologic stage, histologic score, and age that biased the study cohort (Wilcoxon rank-sum test). The patients were monitored every 3–4 months for the 1st year after surgery, at least semiannually for the next 2 years, and annually thereafter, by use of PSA measurements and digital rectal examinations. In our series, no patient with undetectable PSA levels had a documented tumor recurrence. Sixteen patients with pT3 tumors (11 with pT3A–B tumors and five with pT3C tumors) underwent immediate adjuvant external radiotherapy (started within 3 months after surgery). In all other patients who received adjuvant therapy (androgen deprivation, chemotherapy, or radiotherapy), this adjuvant treatment was introduced only after a significant rise in serum PSA level was achieved (>1 ng/mL).

Immunohistochemistry

Antibodies and immunodetection of BSP. One tissue block per patient that contained the most representative tumor-bearing areas was selected after we considered the capsular status (pathologic stage) and the Gleason score stated in the pathology report. For example, when a prostate tumor was reported with a Gleason score 7 (grade 4 + grade 3) and a pathologic stage T3, the most representative block was selected according to these tumor characteristics and thus harbored both grades 3 and 4 as well as foci of extracapsular extension. Using two rabbit polyclonal antibodies (LF83 and LF100), we examined the expression of BSP by the immunoperoxidase technique. These antibodies were raised against synthetic peptides of human BSP (LF83, residues 277–

294; LF100, residues 129–140) *(20)*. Both antisera have been checked previously for reactivity by western blotting and shown to react only with BSP *(20)*. Immunoperoxidase staining was performed by use of the ABC Vectastain Elite kit (Vector Laboratories, Inc., Burlingame, CA) according to the supplier's directions. Briefly, tissue sections were deparaffinized in xylene and rehydrated. After the endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 minutes, the slides were incubated with normal goat serum (1 : 20) to block the nonspecific serum-binding sites. Anti-BSP LF83 at a dilution of 1 : 1000 or anti-BSP LF100 at a dilution of 1 : 1500 was applied and incubated overnight at 4 °C, followed by incubation with a biotinylated goat anti rabbit antibody (1 : 200) (30 minutes) and the avidin–biotin–peroxidase complex (30 minutes). After each incubation, slides were washed three times with phosphate-buffered saline (PBS) (10 m*M* sodium phosphate and 0.9% NaCl [pH 7.5]). Peroxidase activity was developed by a solution of 4 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Vel, Leuven, Belgium) dissolved in 10 mL of PBS and 0.03% H_2O_2. The DAB solution was filtered, and the sections were incubated for 4 minutes. Finally, Carazzi's hematoxylin was used to counterstain the slides, which were then dehydrated and mounted. Immunoreactivity with the LF83 antibody was evaluated for all 180 tissue sections. Detection of BSP expression with the LF100 antibody was attempted in 30 unselected specimens. Control experiments included omission of the first antibody. For the LF83 antibody, preincubations with a 100 *M* excess of the corresponding peptide or an unrelated peptide (synthetic peptide of bovine osteonectin LF54, residues 27–56) were performed prior to the antibody's use in the immunoperoxidase assay.

Evaluation of immunohistochemical staining. Two independent observers, who had no knowledge of the patients' outcome, reviewed the immunohistochemically stained sections. When present, normal and hyperplastic glands as well as prostatic intraepithelial neoplasia (PIN) lesions adjacent to the tumor were also evaluated for anti-LF83 and anti-LF100 immunoreactivity. In a primary evaluation, scoring

of the staining was arbitrarily done according to the intensity of the staining (0, 1+, 2+, or 3+) and the percentage of positive neoplastic glands within the tumor (0%, <10%, 10%–33%, 34%–66%, or 67%–100%). When the tumors showed some heterogeneity in the intensity of the staining, the scoring of the intensity of the staining was assessed according to the staining of the most positive tumor cells, when their estimated percentage represented at least 30% of the total positive tumor cell area. In a secondary analysis, tumor samples were subclassified with regard to immunostaining intensity and extent according to similar graded scales that ranged from 0 to 3. For staining intensity, the scale used was identical to that described above. For staining extent, 0 represented samples in which BSP expression was undetectable, 1 denoted samples in which up to 33% of the glands exhibited a detectable level of anti-BSP immunoreactivity, 2 signified those with detectable staining in more than one third up to two thirds of the tumor cells, and 3 represented those in which more than two thirds of the tumor cells expressed a detectable level of BSP expression. The results obtained with the two scales were multiplied together, yielding a single scale with steps of 0, 1+, 2+, 3+, 4+, 6+, and 9+, where 0 was considered to be negative staining, 1+ and 2+ were considered to be low staining, 3+ and 4+ were considered to be medium staining, and 6+ and 9+ were considered to be high staining. This multiplied scale subclassification system is similar to that previously described by Sjögren et al. *(21)*.

In Situ Hybridization of BSP mRNAs

Probes. The template used was the full-length human BSP complementary DNA (cDNA) B6–5g *(7)* cloned in the Stratagene pBluescript SK vector (Stratagene, La Jolla, CA). After linearizing the plasmid with the appropriate restriction enzymes (*Bam*HI and *Hin*dIII for antisense and sense probes, respectively), we obtained a digoxigenin (DIG)-labeled single-stranded antisense RNA probe by use of T7 RNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany) and a DIG RNA-labeling kit (Boehringer Mannheim GmbH). Similarly, a sense DIG-labeled RNA probe was prepared for negative control experiments by use of T3 RNA polymerase and the same labeling kit.

Harvesting and preparing human prostate cancer tissue. Fresh samples of normal and malignant human prostate tissues were obtained from radical prostatectomy specimens according to the method described by Wheeler and Lebovitz *(22)*. Briefly, in the operating room, prostate tissue samples were taken from the peripheral zone by use of a 6- or 8-mm-diameter punch biopsy instrument. These samples were immediately placed in Tissue-Tek OCT compound (Miles Inc., West Haven, CT), snap-frozen in liquid nitrogen, and stored at –80 °C until sectioning. The tissue sections (8 μm thick) used for *in situ* hybridization were layered onto glass slides coated with 3-aminopropyltriethoxysilane (Sigma-Aldrich S.A., Bornem, Belgium). Serial sections from each specimen were allowed to air-dry prior to fixation for 15 minutes in 4% paraformaldehyde in PBS. After being washed in PBS, the slides were dehydrated through a series of incubations in 30%, 50%, and 70% ethanol, for 5 minutes

each. The sections were then stored in 70% ethanol at 4 °C until use for *in situ* hybridization. A serial section from each sample was examined by use of hematoxylin–eosin staining to select normal areas, PIN lesions, and adenocarcinomas. Another serial section was processed for immunohistochemistry by use of the anti-BSP LF83 antibody at a dilution of 1 : 1000.

BSP-mRNA detection by *in situ* hybridization. The presence of BSP-specific transcripts in primary prostate cancer samples was demonstrated by a non-radioactive *in situ* hybridization technique *(23–25)*. After a hydration step in PBS, tissues were acetylated for 10 minutes in freshly prepared 0.25% acetic anhydride in 0.1 *M* triethanolamine (pH 8.0) and subsequently rinsed in 0.1 *M* glycine and 0.1 *M* Tris (pH 7.0) for 10 minutes. The sections were then dehydrated in increasing ethanol concentrations and air-dried. The hybridization reaction was carried out at 55 °C overnight in hybridization buffer (50% deionized formamide, 5× standard saline citrate [SSC], 10% dextran sulfate, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate [SDS], and 100 μg/mL single-stranded DNA). Approximately 30 μL of hybridization buffer containing 30 μg/mL of DIG-labeled cDNA probes (sense or antisense) was applied to each section. After hybridization, the sections were washed four times (3 minutes each) in 2× SSC and 0.1% SDS and then two times (15 minutes each) in 0.1× SSC and 0.1% SDS at 55 °C. The tissues were then treated with 10 mg/mL ribonuclease A (Boehringer Mannheim GmbH) at 37 °C for 15 minutes. After being washed with 2× SSC for 2 minutes, the sections were incubated with a sheep alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim GmbH) for 30 minutes. The alkaline phosphatase reaction was developed by addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium for 90 minutes. Finally, sections were counterstained with methyl green and mounted under coverslips for microscopic examination. The intensity of the signal in the carcinoma cells and adjacent normal glands when present was graded on a scale of 0 to 3+ (0, no reactivity; 1+, weak reactivity; 2+, moderate reactivity; 3+, strong reactivity).

Statistical Analysis

Biochemical progression was defined as an increasing total serum PSA level of 0.5 ng/mL or more. To fulfill these criteria, at least two successive determinations of increased total serum PSA levels were required to define biochemical failure. During the period from 1987 through March 1995, PSA testing was performed with the use of the Hybritech Monoclonal Assay (Hybritech Europe, Liège, Belgium), and the level of detectability for this test was greater than or equal to 0.5 ng/mL. In the following months, through March 1997, the PSA-UltraSensitive MAGIA® Merck test (Merck-Clevenot, Nogent-sur-Marne, France) was used, and the threshold of detectability was lowered to greater than or equal to 0.1 ng/mL. The actuarial probability of remaining free of biochemical progression was estimated by Kaplan–Meier analysis *(26)*. The progression-free rates were compared with the use of the logrank test. CIs for the 1- and 3-year progression-free rates were computed on the log-survival

scale. Five-year probabilities were not reported because of the small number of men who were followed for this interval of time. The Mantel–Haenszel 1-degree-of-freedom correlation test was used to assess whether pathologic findings (pathologic stage and Gleason score) were significantly associated with LF83 immunostaining intensity and extent. These variables were analyzed as ordered variables and were coded 1, 2, and 3 for pT2, pT3A–B, and pT3C, respectively; 1, 2, and 3 for Gleason scores 2–4, Gleason scores 5–7, and Gleason scores 8–10, respectively; 0, 1, 2, and 3 and 0, 1, 2, 3, and 4 for LF83 staining intensity and extent, respectively, according to the scale described in the previous section entitled "Evaluation of Immunohistochemical Staining." To account for more than one prognostic variable simultaneously, the clinical and histologic parameters (stage and score) and BSP expression were evaluated by use of a multivariate Cox proportional hazards model *(27)* (with stepwise procedure) to determine which variables were independently correlated with progression. The data were consistent with the assumptions of Cox proportional hazards analysis. The dichotomization of LF83 immunostaining intensity and that of the multiplied scale were chosen as 0 and 1+ versus 2+ and 3+ and were specified prior to multivariate analysis. All statistical tests were two-tailed, and *P*< .05 was considered statistically significant. The analyses were performed with statistics software packages (SAS Institute Inc., Cary, NC; and S-Plus, MathSoft Inc., Seattle, WA).

Results

The expression of BSP was evaluated by immunoperoxidase staining in paraffin sections of primary prostate cancer obtained from 180 patients who had undergone a radical prostatectomy for localized prostate cancer. Epithelial cells from normal glands exhibited no or a low, but detectable, level of BSP (Fig. 1, C and D). We observed that, in some PIN glands, when present, BSP expression was increased compared with normal cells (Fig. 1, F). Of the 180 prostate cancer lesions studied, 142 (78.9%) expressed detectable levels of BSP (1+, 2+, or 3+) (Fig. 1, C, D, E, and G). The immunoreactivity was mainly cytoplasmic. In most lesions, BSP expression was heterogeneous within the same tumor (Fig. 1, H). BSP was also detected with the use of the LF100 polyclonal antibody in 30 unselected prostate cancer samples. Both anti-BSP antibodies (LF83 and LF100) generated the same degree and pattern of specific staining (data not shown). Positive staining was abolished by omission of the primary antibodies and was significantly reduced by pre incubation with a 100 *M* excess of the corresponding synthetic peptide (Fig. 1, A
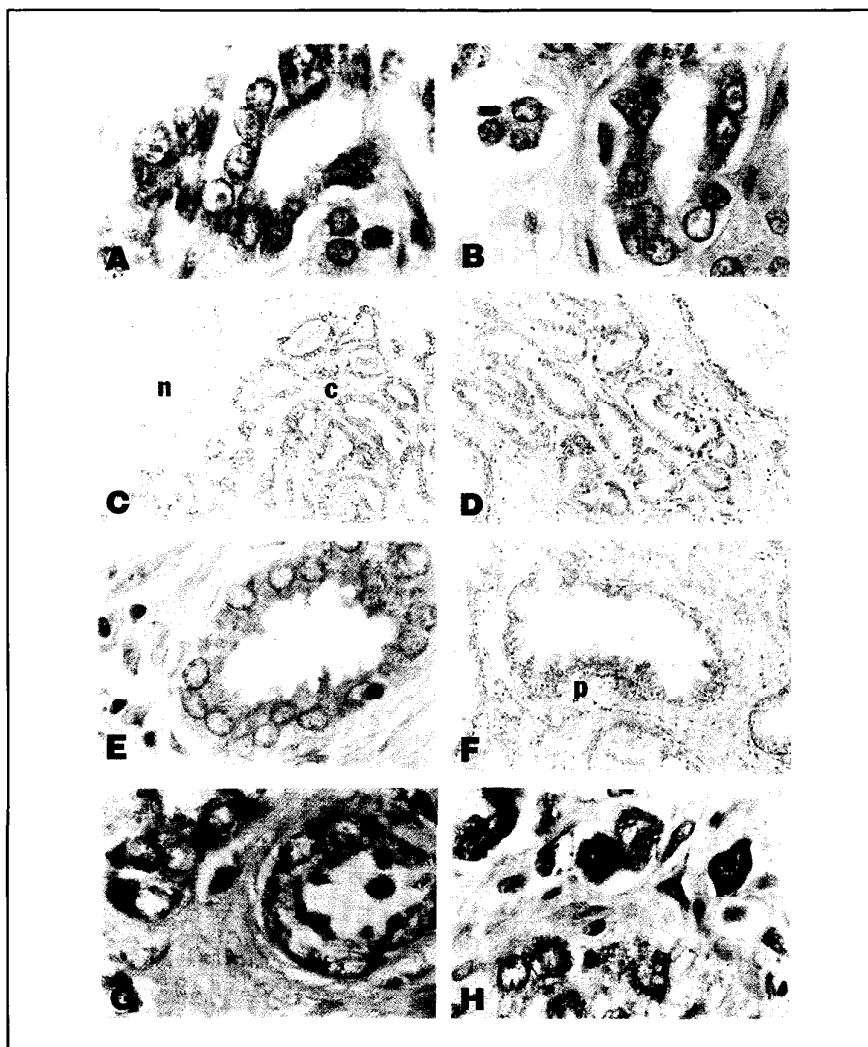
Fig. 1. Immunodetection of bone sialoprotein (BSP) in human prostate cancer by immunoperoxidase staining. Paraffin-embedded tissue sections were immunostained with LF83 polyclonal antibody and counterstained with hematoxylin as described in the "Materials and Methods" section. Level 1+ expression of BSP is not shown. A) Representative prostate adenocarcinoma expressing BSP at a 2+ level. B) Preincubation of LF83 anti-BSP antibody with its corresponding peptide results in a significant decrease of immunostaining when applied to the same lesion as shown in A. C) Moderately differentiated prostate carcinoma (c) exhibiting a 3+ BSP immunostaining. Note the absence of detectable level of BSP expression in normal prostate glands (n). D) Well-differentiated prostate cancer showing no BSP immunostaining. E) Representative prostate cancer gland exhibiting a 2+ BSP staining. F) Focal prostatic intraepithelial neoplasia area (p) with 2+ BSP expression. G) Homogeneous 3+ expression of BSP in moderately differentiated prostate cancer glands. H) Poorly differentiated prostate cancer showing heterogeneous 3+ BSP immunostaining.

and B). No significant difference in LF83 was observed after preincubation of the antibody with a 100 M excess of an unrelated peptide (data not shown).

The intensity of BSP expression significantly correlated with the extension of the primary tumor (from organ confined [pT2] to extracapsular [pT3A–B] to seminal vesicle-invading tumors [pT3C]) (Mantel–Haenszel test, $P = .04$), but it was not significantly associated with tumor differentiation, expressed according to the Gleason score (Mantel–Haenszel

test, $P = .10$). We found a borderline association between the extension of the tumor and the extent of the LF83 staining (Mantel–Haenszel test, $P = .052$). In the multiplied scale system, which takes into account both the heterogeneous pattern and the intensity of BSP expression, a significant association was observed between subclass scores (0 versus 1–2 versus 3–4 versus 6–9) and the pathologic stage of the prostate tumors (Mantel–Haenszel test, $P = .012$).

Monitoring measurements of postop-

erative total serum PSA levels were available for 164 of the 180 patients. The 1- and 3-year overall biochemical nonprogression rates were 87.5% (95% CI = 82.3%–92.9%) and 73.5% (95% CI = 65.6%–82.4%), respectively. The 164 patients were subclassified into two groups according to the intensity of BSP expression: group 1, low BSP expression (staining intensity 0 and 1+); group 2, high BSP expression (staining intensity 2+ and 3+). This dichotomization of LF83 immunostaining was specified before statistical analyses were performed. Actuarial biochemical progression-free curves of these two groups differed significantly; the risk of PSA relapse was higher in patients whose tumors expressed high levels of BSP (group 2, 36.7% [95% CI = 23.4%–47.7%]) than in patients whose tumors expressed low, but detectable, levels of the protein (group 1, 12.1% [95% CI = 2.3%–20.8%]) (logrank test, $P = .0014$) (Fig. 2, A). An increased extent of LF83 immunostaining was also associated with a significantly higher PSA relapse rate (logrank test, $P<.001$). The data were analyzed considering the different subclasses obtained from the more complex multiplied scale grading system (intensity × extent of staining). We observed significant differences between PSA progression-free rates of the different categories from the four-grade scale (0 versus 1–2 versus 3–4 versus 6–9) (logrank test, $P = .003$) (Fig. 2, B). In the group of patients with pathologically confined tumors (pT2), significant differences were found in relapse-free survival rates between patients with tumors expressing low levels of BSP and those with tumors expressing high levels of BSP, with a worse prognosis for patients with tumors highly positive for BSP (logrank test, $P = .014$). The 3-year PSA-relapse frequency was 2.3% (95% CI = 0%–6.6%) in the group of patients with tumors expressing low levels of BSP compared with 14.1% (95% CI = 0%–26.5%) in the group of patients with tumor expressing high levels of BSP (Fig. 2, C). It is interesting that none of the patients with pT2 tumors that did not exhibit BSP (staining intensity 0) had a PSA relapse.

Estimated 3-year biochemical progression-free rates for well-differentiated (Gleason scores 2–4), moderately differentiated (Gleason scores 5–7), and poorly differentiated (Gleason scores 8–10)
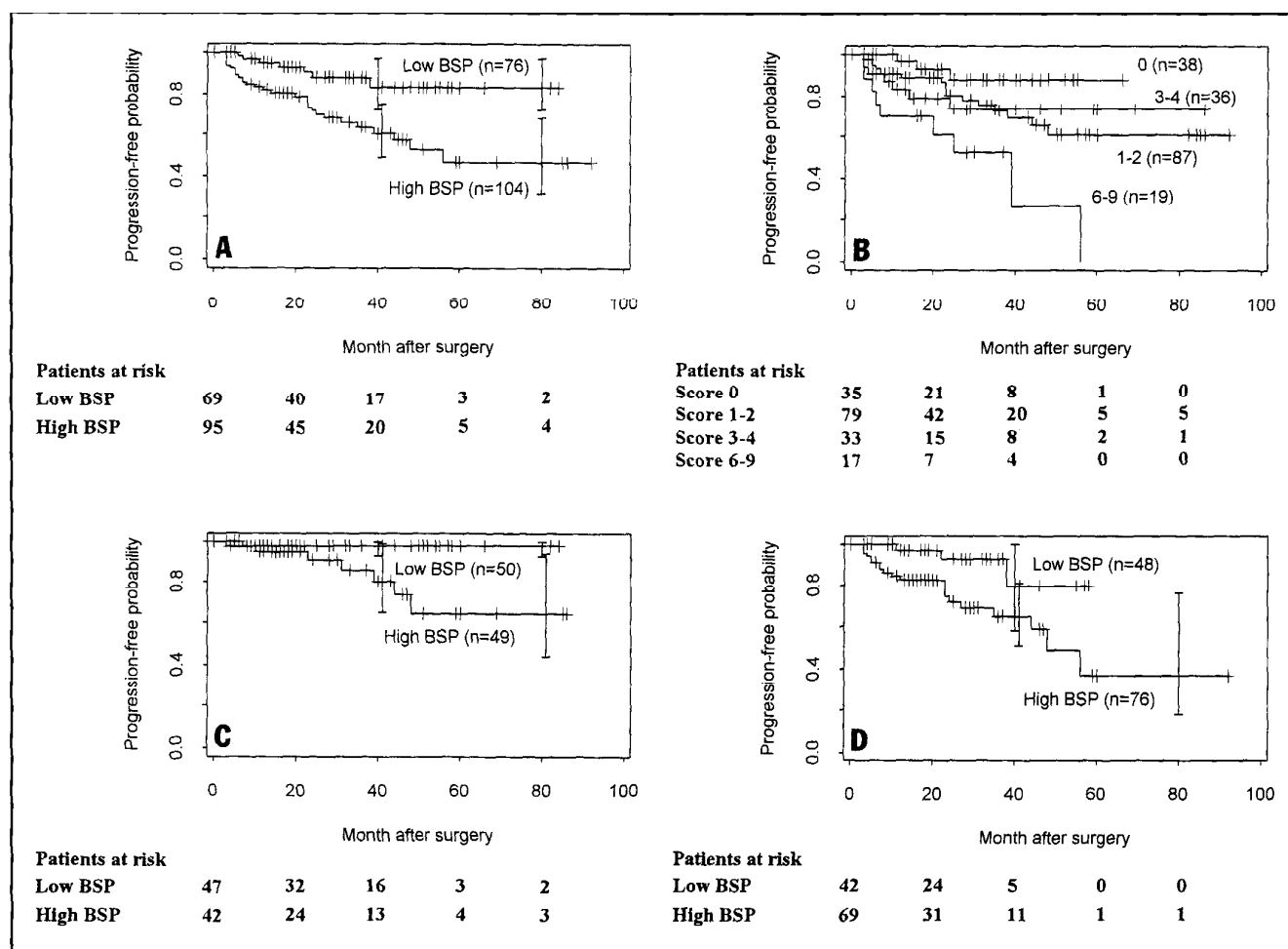
Fig. 2. Biochemical progression-free rates in patients with clinically localized prostate cancer. Biochemical progression was defined as an increasing total serum prostate-specific antigen (PSA) level of 0.5 ng/mL or more. Tick marks on the curves indicate censored patients. Representative 95% confidence intervals (CIs) are shown in **panels A, C,** and **D.** Patients at risk for each category of bone sialoprotein (BSP) immunostaining are shown below each panel. All reported P values are two-sided. **Panel A:** Actuarial probability of freedom from progression according to BSP expression for 180 patients with clinically localized prostate cancer who were treated by radical prostatectomy. We observed a significantly higher 3-year PSA relapse rate in patients with tumors exhibiting a high level of BSP expression (staining intensity 2+ or 3+) (36.7% [95% CI = 23.4%–47.7%]) than in patients whose tumors expressed no or low detectable levels of BSP (staining intensity 0 or 1+) (12.1% [95% CI = 2.3%–20.8%]) (logrank test, P = .0014). **Panel B:** Actuarial biochemical progression-free disease according to the intensity and the extent of BSP expression. The whole population of 180 patients was categorized according to anti-BSP immunostain-

ing intensity and extent following a multiplied 4-grade scale described in the "Materials and Methods" section. For legibility purpose, the additional lines that represent 95% CIs are not shown. Three-year progression-free estimates were 87.9% (95% CI = 75.6%–100%), 71.9% (95% CI = 60.2%–86%), 74.3% (95% CI = 59.1%–93.5%), and 52.3% (95% CI = 31.3%–87.2%) in patients with no detectable BSP staining (0), low staining (1–2), medium staining (3–4), and high staining (6–9), respectively (logrank test, P = .003). **Panel C:** Actuarial PSA relapse rates in the 99 patients with pT2 tumors. A higher biochemical progression rate was observed in patients with pT2 tumors that expressed high levels of BSP expression (staining intensity 2+ or 3+) than in those with tumors expressing no or low levels of the protein (staining intensity 0 or 1+) (logrank test, P = .014). **Panel D:** High expression of BSP (staining intensity 2+ or 3+) by the tumor cells was associated with a higher risk of biochemical progression in patients with moderately differentiated tumors (Gleason scores 5–7) (logrank test, P = .011).

cancers were 95% (95% CI = 85.9%–100%), 74.7% (95% CI = 64.6%–86.3%), and 38.5% (95% CI = 21.3%–69.7%), respectively (logrank test, $P<.0001$). In the large group of 124 patients with moderately differentiated tumors, significant differences were observed in biochemical relapse-free rates between patients with prostate tumors that exhibited a low level of LF83 immunoreactivity (group 1) and those with tumors that were highly positive for BSP (group 2) (logrank test, P = .011). The 3-year relapse-free survival rate in the first group was 92.8% (95% CI = 83.6%–100%), as opposed to 64.6% (95% CI = 51.3%–81.4%) in the second group (Fig. 2, D).

Sixteen patients with extracapsular disease (pT3A–B–C) had undergone immediate adjuvant radiation therapy following radical prostatectomy. To exclude any bias that might affect the survival results in the group of patients with pT3A–B–C cancers who were irradiated and the group of patients with pT3A–B–C tumors who were not irradiated, we compared the groups with regard to clinical or pathologic stage, histologic score, and age; however, we did not find any significant differences (Wilcoxon rank-sum test). Time to PSA relapse was then evaluated in these two populations of patients with pT3A–B–C cancers (immediate adjuvant

radiotherapy versus no immediate adjuvant treatment). Three-year progression-free rates were 62.3% (95% CI = 40.1%–96.7%) and 45.4% (95% CI = 31.2%–66%) for patients with and without adjuvant radiation therapy, respectively (logrank test, P = .43). Because we observed no difference in intensity of BSP expression between those two groups of patients (Wilcoxon rank-sum test, P = .26), we could conclude that our estimate of the association between BSP and PSA relapse was not altered by adjuvant radiotherapy.

A Cox proportional hazards model for multivariate analysis was developed to investigate whether prognostic information generated by BSP expression was positively or negatively confounded by other commonly used prognostic markers. Covariates included in the model were BSP staining intensity, BSP staining extent, BSP staining extent × intensity, clinical stage, pathologic stage, and Gleason score. Multivariate analysis identified (in order of significance) Gleason score (P<.0001), intensity of BSP expression (P<.0005), and pathologic stage (P<.05) as strong independent predictors of PSA recurrence, with risk ratios of 1.7 (95% CI = 1.3–2.2), 1.8 (95% CI = 1.2–2.7), and 2.8 (95% CI = 1.2–6.5), respectively.

To evaluate the additional prognostic information associated with the detection of BSP, we used Cox's proportional hazards model to estimate the 1-year and 3-year progression-free survival rates based on the Gleason score alone and after combining the Gleason score and BSP intensity (Table 2). The results of this analysis were particularly striking in the groups of patients with Gleason score 5–7 and score 8–10 tumors. For example, if the Gleason score was the only covariate entered in the model, the analysis estimated a 38%

progression-free survival rate at 3 years for the group of patients with Gleason score 8–10 tumors. When we added BSP intensity to the multivariate analysis, important differences in the 1-year and 3-year progression-free rates were observed. A comparison between the progression-free rates of the entire group of patients with Gleason score 8–10 tumors (67.6% and 38% at 1 and 3 years, respectively) and those of two subgroups of patients with score 8–10 tumors that expressed low (0 or 1+) or high (2+ or 3+) levels of BSP yielded significant differences. In extenso, the estimated risk of relapse for a patient with a Gleason score 8–10 prostate tumor in this series was 62%. If this patient's tumor expressed low levels of BSP, the relapse risk was reduced to 34.6% and was nearly the same as that of a patient with a Gleason score 5–7 tumor that expressed high levels of the protein (32.8%). Conversely, if high levels of BSP were detected in the cells of a score 8–10 tumor, the estimated risk of PSA relapse was increased to 74.9%. These data clearly show that BSP detection can significantly contribute to a more accurate identification of patients with poor prognosis.

In situ hybridization with sense and antisense riboprobes was performed on eight representative prostate cancer specimens in order to demonstrate the presence of BSP mRNA in the prostate carcinoma cells. Sense and antisense BSP riboprobes were prepared and applied to serial prostate cancer sections. The expression of BSP mRNA was high in prostate cancer glands as compared with that in normal glands, which exhibited no or a low level of the transcripts (not shown). BSP mRNA was also detected in some PIN lesions. The level of expression of BSP mRNA was usually consistent with the

amount of the protein detected by immunoperoxidase. Control experiments in which the sense riboprobe was used showed no specific reactivity (data not shown).

Discussion

The search for reliable predictors of prostate cancer progression is a major and urgent research challenge for the next few years. A wide discrepancy exists between the steadily increasing number of sub-clinical malignant lesions diagnosed and the prevalence of those lesions that actually will progress to clinically overt disease (28–33). There is, therefore, an essential need for better pretherapeutic assessment of the aggressiveness of these lesions. Prostate cancer progresses along a predictable pathway: invasion of the draining lymph nodes through the lymphatic routes and colonization of the skeleton through hematogeneous dissemination (34). Bone metastases are a constant feature of advanced prostate cancer and create major morbidity (35–37). Until now, little has been known about the mechanisms that lead circulating metastatic prostatic cells to target bone as a preferred site for the successful initiation of a secondary lesion. Potential clues that could help to unveil the mechanisms of bone metastasis formation are provided by the recent observation that several osteotropic cancers, including breast and lung carcinomas as well as myeloma, ectopically express BSP (6,14,15,20). Our finding that BSP expression in primary breast cancer correlates with both the development of bone metastases and patient survival (16,17) suggests that this protein could play a role in the determination of the osteotropic phenotype of metastatic cancer cells. It is now generally acknowledged that cross talk between cancer cells and their host tissues is critical for the initiation of a metastasis. We have recently proposed that ectopic BSP expression by circulating malignant cells could favor their interactions with mineralized bone matrix, e.g., mediate the cells' attachment to hydroxyapatite crystals. The demonstration that breast cancer cells that metastasize to bone express the integrin $\alpha_v\beta_3$, a cell surface receptor that uses BSP as a ligand, is in agreement with our hypothesis (38–43). Further support for

Table 2. Progression-free probabilities estimated according to the Gleason score and bone sialoprotein (BSP) intensity

Gleason scores*	Progression-free		BSP intensity	Progression-free	
	At 1 y, %	At 3 y, %		At 1 y, %	At 3 y, %
2–4	96.8	92.3	0 to 1+	98.6	96.5
			2+ to 3+	95.5	89.2
5–7	89.3	75.7	0 to 1+	95.2	88.5
			2+ to 3+	85.3	67.2
8–10	67.6	38.0	0 to 1+	84.4	65.4
			2+ to 3+	57.5	25.1

*2–4, well differentiated; 5–7, moderately differentiated; 8–10, poorly differentiated.

this concept is provided by the recent report that BSP-derived peptides bearing the RGD (i.e., arginine–glycine–aspartic acid) sequence can inhibit the adhesion of breast cancer cells to bone (44). If ectopic expression of BSP is associated with the osteotropic phenotype, it would be expected that this bone matrix protein would also be detected in the cancerous cells originating from other osteotropic cancers. Indeed, lung (14) and breast (17) cancers were frequently found to express BSP. In this study, we have demonstrated that BSP expression is also a common phenotypic feature of human prostate cancers, the second most osteotropic cancer known. In situ hybridization experiments demonstrated that BSP mRNA was expressed in prostate carcinoma cells that exhibited high levels of immunoreactivity with the anti-BSP antibodies.

The level of BSP expression was significantly associated with the pathologic stage of the tumor. In contrast, only a weak trend of association was observed between BSP expression and another commonly accepted prognostic marker, the histologic score of the tumor (45–50). Total serum PSA is unanimously recognized as the most important and clinically significant tumor marker available to date. Since 1987, PSA testing has been commonly used to monitor the patient's response to prostate cancer therapy. An increasing level of PSA after curative treatment is, in most cases, the first indicator of recurrence. In this study, we demonstrate that a high level of BSP expression is independently associated with a higher risk of PSA relapse in patients who have undergone a radical prostatectomy for clinically confined prostate cancers. Patients with prostate tumors of intermediate Gleason scores 5–7 (moderately differentiated) represent the largest group with prostate cancer. These patients represent the "gray zone" cohort because disease recurrence may be highly variable from one patient to another. One interesting finding in this study is the observation that the level of BSP expression provides valuable additional prognostic information in this group of patients. Indeed, the detection of high levels of the protein significantly identifies a group of patients with a higher risk of biochemical progression. Because of the long follow-up that is necessary, it is not clear yet whether these results are predictive of the clinical out-

come in terms of survival and, more particularly, of the development of bone metastases. PSA relapse in the group of patients with pathologically confined tumors (pT2) is usually low and varies from 6% to 18% at 5 years, depending on the cut point used to define biochemical progression (49,51,52). Our data reveal that up to 17% of patients with pT2 prostate tumors demonstrate PSA elevation at 5 years (results not shown). We observed that patients with pT2 tumors that express high levels of BSP show a significantly higher 3-year PSA recurrence rate than patients with pT2 tumors that express no or low levels of the protein. It is interesting that none of the patients with pT2, BSP-negative prostate cancers had PSA relapse on follow-up. We might then hypothesize that, in the group of patients with pT2 cancers who were treated and believed to be cured by radical prostatectomy, ectopic BSP expression is a required event for disease progression. If studies performed on larger cohorts of patients confirm these data, then BSP detection could be used to identify the risk that a patient's tumor will progress.

Invasion of the seminal vesicles (stage pT3C) is associated with a high rate of disease progression. For this group of patients, the prognosis is nearly as poor as that for patients with lymph node metastases (45,49,53–55). In our series, we observed that, within 3 and 5 years of follow-up, 54% and 70% of patients, respectively, with pT3C tumors had PSA recurrence (results not shown). In the group of patients with pT3C tumors, we observed a high rate of BSP expression by the tumor cells, with 96% of the pT3C prostate specimens exhibiting detectable levels of the protein (results not shown).

Finally, we showed that BSP detection, when combined with the Gleason score, may provide clinicians with valuable additional prognostic information to evaluate with increased accuracy the outcome for a patient who has undergone a radical prostatectomy for clinically confined prostate cancer.

In conclusion, to our knowledge, we demonstrate for the first time in this study that BSP is ectopically expressed in most human prostate cancers and that a high level of BSP expression is associated with a higher risk of biochemical progression after radical prostatectomy for localized prostate cancer. The best pretherapeutic

assessment of prostate tumor aggressiveness will probably result from the detection of biologic markers associated with the invasive and metastatic phenotype of the tumor cells. This challenge will be met when use of such markers allows an accurate prediction of the risk of tumor progression for an individual patient. It is likely that a combination of several markers will be required to provide the clinicians with sufficient confidence to decide which patient would benefit from watchful waiting, from immediate radical prostatectomy, or from other treatments.

We have recently demonstrated that the 67-kd laminin receptor (67LR), a protein associated with the invasive phenotype of malignant cells, was an independent prognostic marker of PSA relapse in patients who have been treated by radical prostatectomy for localized prostate cancer (56). In the near future, we shall determine whether the combination of the two biologic markers (67LR and BSP) offers additional value in the prognostic evaluation of prostate cancer lesions. Furthermore, with the demonstration that expression of BSP is a common feature in prostate cancer, a highly osteotropic cancer, we have an accumulation of evidence that incriminates this bone matrix protein in the process of bone metastasis formation. Our new challenge now is to determine how and when BSP expression is turned on in the oncogenic process and to investigate the specific potential molecular roles that this bone matrix protein may play during bone colonization. It is tempting to speculate that the identification of these mechanisms could lead to a putative treatment to prevent bone metastases.

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Notes